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Stem Cell Research (2015) 15, 1–13

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A matter of identity — Phenotype and differentiation potential of human somatic stem cells

S.E.P. New[a,1], C. Alvarez-Gonzalez[b,c,1], B. Vagaska[a,1], S.G. Gomez[d], N.W. Bulstrode[e], A. Madrigal[b,c], P. Ferretti[a,*]

[a] Stem Cells and Regenerative Medicine, UCL Institute of Child Health, London WC1N 1EH, UK
[b] Anthony Nolan Research Institute, London NW3 2QG, UK
[c] UCL Cancer Institute, LondonWC1E 6DD, UK
[d] Anthony Nolan Cell Therapy Centre, Nottingham NG11 8NS, UK
[e] Department of Plastic Surgery, Great Ormond St. Hospital for Children NHS Trust, London WC1N 3JH, UK

Received 8 December 2014; received in revised form 13 April 2015; accepted 18 April 2015
Available online 25 April 2015

Abstract
Human somatic stem cells with neural differentiation potential can be valuable for developing cell-based therapies, including treatment of birth-related defects, while avoiding issues associated with cell reprogramming. Precisely defining the "identity" and differentiation potential of somatic stem cells from different sources, has proven difficult, given differences in sets of specific markers, protocols used and lack of side-by-side characterization of these cells in different studies. Therefore, we set to compare expression of mesenchymal and neural markers in human umbilical cord-derived mesenchymal stem cells (UC-MSCs), pediatric adipose-derived stem cells (p-ADSCs) in parallel with human neural stem cells (NSCs). We show that UC-MSCs at a basal level express mesenchymal and so-called "neural" markers, similar to that we previously reported for the p-ADSCs. All somatic stem cell populations studied, independently from tissue and patient of origin, displayed a remarkably similar expression of surface markers, with the main difference being the restricted expression of CD133 and CD34 to NSCs. Expression of certain surface and neural markers was affected by the expansion medium used. As predicted, UC-MSCs and p-ADSCs demonstrated tri-mesenchymal lineage differentiation potential, though p-ADSCs display superior chondrogenic differentiation capability. UC-MSCs and p-ADSCs responded also to neurogenic induction by up-regulating neuronal markers, but crucially they appeared morphologically immature when compared with differentiated NSCs. This highlights the need for further investigation into the use of these cells for neural therapies. Crucially, this study demonstrates the lack of simple means to distinguish between different cell types and the effect of culture conditions on their phenotype, and indicates that a more extensive set of markers should be used for somatic stem cell characterization, especially when developing therapeutic approaches.
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Abbreviations: p-ADSC, pediatric adipose tissue derived stem cells; NSC, neural stem cells; UC-MSC, umbilical cord derived stem cells

* Corresponding author at: Stem Cells and Regenerative Medicine Section, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK.
E-mail address: p.ferretti@ucl.ac.uk (P. Ferretti).
[1] Joint first authors, shared contribution.

http://dx.doi.org/10.1016/j.scr.2015.04.003
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Introduction

There are several challenges to producing cell-based therapies of the quality, safety and scale needed for clinical use. The use of somatic stem cells offers the advantage of avoiding the issue of tumorigenicity and costly reprogramming linked to the use of ES and iPS (Ben-David and Benvenisty, 2011; Bianco et al., 2013), as well as offering the possibility of using minimally manipulated autologous stem cells and thereby preventing any risk of rejection or Graft-versus-Host-Disease seen with the use of allogeneic tissues and cells. Important factors to be considered in the development of cell transplantation therapies are availability and differentiation potential of the stem cell type selected, and the use of allogeneic versus autologous stem cells.

It has long been known that mesenchymal stem cells (MSCs) are capable of differentiation along the mesenchymal lineages, forming adipose tissue (adipocytes), cartilage (chondrocytes) and bone (osteoblasts) (Pittenger et al., 1999, 2000). MSCs can be isolated from a number of tissues, including but not limited to bone marrow, adipose tissue, and, umbilical cord blood and matrix (Pittenger, 2008; Wang et al., 2004; Zuk et al., 2001). Bone marrow derived MSCs (BMSCs) have been the most widely studied of the MSCs, however due to the invasive nature of their isolation, much attention has been recently paid to other MSCs. Umbilical cord matrix, umbilical cord blood and adipose tissue are byproducts of certain procedures and previously thought of as biological waste, thus preventing much of the ethical problems associated with the use of other stem cells and making them ideal candidates for use in stem cell therapies. We have previously shown the high plasticity of pediatric adipose derived-stem cells (p-ADSC) and their ability to differentiate towards the mesenchymal and neurogenic lineages (Guasti et al., 2012). However, it has since been reported to be easier to isolate MSCs with a high purity from Wharton's Jelly, than from bone marrow or adipose tissue (Liu et al., 2014), therefore making this source extremely attractive. There is potential for stem cells to be employed to treat childhood neurological disorders, as well as to be used to further our understanding of the developing nervous system and the pathological processes that underlie these conditions. The therapeutic benefits of MSCs have been demonstrated in animal models recapitulating ischemic stroke, spinal cord injury and Parkinson's disease (Ding et al., 2007; Yang et al., 2008). Despite this clinical trials have not proved as successful (Chen et al., 2013; Dalous et al., 2012; Mazzini et al., 2006; Prasongchean and Ferretti, 2012; Uccelli et al., 2011).

The aim of this study was to comprehensively compare, for the first time, pediatric p-ADSCs and UC-MSCs, characterizing their marker expression and differentiation capability including their neurogenic potential alongside human neural stem cells (NSCs). We establish, for the first time, the similarity in surface marker expression profiles between these three human somatic stem cell types despite their origins. The use of animal-based products in the culturing of cells for therapeutic purposes is undesirable (Tekkatte et al., 2011). Here we assessed whether the use of cord blood plasma instead of fetal calf serum may provide a simple way of avoiding the use of animal based products in UC-MSC cultures. In our hands although UC-MSCs had a lower potential to differentiate towards the mesenchymal lineages, both UC-MSCs and p-ADSCs demonstrated similar upregulation of neural markers after induction, as well as a significant change in morphology. Despite this, their immaturity in terms of neurogenicity was apparent when compared side-by-side with NSCs. The differences in preferential differentiation of somatic stem cells reported here and the medium used may have important bearing on the use of these somatic stem cells for therapeutic purposes.

Materials and methods

Materials

All chemicals were from Sigma-Aldrich (St Louis, USA), unless otherwise stated.

Cell growth and differentiation

All procedures involving human tissue were carried out in accordance to the Human Tissue Act 2006. Cells were grown in humidified incubators at 37 °C with 5% CO_2.

Human umbilical cord mesenchymal stem cells (UC-MSCs)

Whole umbilical cords were collected from consenting mothers with healthy full-term pregnancies. Umbilical cord mesenchymal stem cells (UC-MSC) were isolated from the Wharton's jelly of the umbilical cord using a modified protocol described previously by Weiss et al. (Weiss et al., 2006) and unless stated otherwise were cultured in high glucose Dulbecco's modified Eagle's medium with GlutaMAX™ (DMEM; Life Technologies) and supplemented with 10% embryonic stem cell-qualified fetal bovine serum (ES-FBS; Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Life Technologies) — termed UC-MSC–FBS. In some experiments UC-MSC were also cultured in umbilical cord blood low enriched plasma, supplemented with EGF and FGF2; these cells are referred to as UC-MSC–CP within the text. Low enriched cord plasma (CP) was prepared by following a protocol modified from Chieregato et al. (Chieregato et al., 2011). Briefly, CP was thawed at 37 °C to promote cell disruption. After thawing, CP was heat inactivated at 56 °C for 30 min, centrifuged at 1000 *g* for 10 min at room temperature to spin down all the particles, and finally the CP supernatant removed and stored at −20 °C prior to use.

Human pediatric adipose tissue-derived stem cells (p-ADSCs)

Abdominal adipose tissue was collected from consenting patients under ethical approval from the Camden and Islington Community Local Research Ethics Committee (London, UK). p-ADSCs were isolated from lipoaspirates of pediatric patients as previously described (Guasti et al., 2012). Isolated p-ADSCs were cultured in high glucose DMEM with GlutaMAX™ and supplemented with 10% ES-FBS and 1% penicillin/streptomycin.

Human neural stem cells (NSCs)

The brains from human embryos between 6 and 10 weeks old were collected through Human Developmental Biology Resource (HDBR) and human NSCs were isolated as

previously described by Sun et al. (Sun et al., 2008). Isolated NSCs were grown as monolayers in DMEM/F12 media with Glutamax (Life Technologies) supplemented with 1% penicillin/streptomycin, 1% N2 supplement, 2% B27 supplement (both Life Technologies), 20 ng/ml human recombinant FGF2, 20 ng/ml human recombinant EGF (both Peprotech), 50 μg/ml BSA fraction V and 5 μg/ml heparin and 10 μg/ml laminin (Sigma).

Adipogenic differentiation

Adipogenic differentiation was induced in confluent cells with DMEM medium containing Glutamax, 1% penicillin/streptomycin, 10% ES-FBS, 1 μM dexamethasone, 10 ng/ml insulin, 500 mM 3-isobutyl-1-methylxanthine and 1 mM rosiglitazone. After 3 weeks, cells were fixed in 10% formalin and analyzed semi-quantitatively with Oil Red staining as previously described (Guasti et al., 2012).

Osteogenic differentiation

Osteogenic differentiation was induced in confluent cells with DMEM medium containing Glutamax, 1% penicillin/streptomycin, 10% ES-FBS, 0.1 μM dexamethasone, 100 μg/ml ascorbate and 10 mM β-glycerophosphate. After 3 weeks, cells were fixed in ice-cold 70% ethanol and analyzed semi-quantitatively with Alizarin Red staining as previously described (Guasti et al., 2012).

Chondrogenic differentiation

Chondrogenic differentiation was induced in confluent cells with DMEM medium containing Glutamax, 1% penicillin/streptomycin, 10% ES-FBS, 0.1 μM dexamethasone, 50 μg/ml ascorbate, 10 ng/ml transforming growth factor (TGF) β1 and insulin, transferrin, selenium. After 3 weeks, cells were fixed in 4% paraformaldehyde and analyzed semi-quantitatively with Alcian Blue staining as previously described (Guasti et al., 2012).

Neural differentiation

Neural differentiation was induced in confluent cells by changing the expansion medium to DMEM with Glutamax supplemented with 1% penicillin/streptomycin, 10% ES-FBS, 10 μM forskolin, 5 mM KCl, 2 mM valproic acid, 1 μm hydrocortisone and 5 μg/ml insulin as described previously (Huang et al., 2007). Cells were either analyzed for gene expression after 3 weeks of differentiation or analyzed for protein expression by immunofluorescence after 2 weeks in this differentiation media. Neural stem cells were differentiated in DMEM/F12 medium with Glutamax and same supplements, omitting the ES-FBS to prevent the differentiation towards an astrocytic phenotype. After 10 days the medium was changed to maturation medium composed of neurobasal medium A supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 2% B27 supplement for another 2 weeks.

Flow cytometry

For cell surface labeling, cells were incubated in 2.5% FBS in PBS blocking solution to prevent any non-specific protein binding. Cells were then incubated with antibodies diluted in blocking solution for 10 min at 4 °C followed by two washes in FACS buffer. For double labeling with intracellular markers the cells were first fixed in 4% paraformaldehyde (PFA) for 20 min at 4 °C in the dark. After staining with surface markers antibodies followed by permeabilization the cells were incubated with intracellular marker antibodies for 30 min at room temperature in dark. For negative controls cells were incubated with the isotype control. The antibodies used and their appropriate dilutions are listed in Supplemental Table 2. BD FACSCalibur TM was used to carry out flow cytometry analysis and data was analyzed using FlowJo 6.4.7 software.

Immunocytochemistry

Cells were fixed in 4% PFA prior to immunocytochemical protein detection followed by incubation with a blocking/permeabilizing buffer (10% FBS, 3% BSA, and 0.2% Triton-X100 in PBS). Primary and secondary antibodies were diluted in blocking buffer as specified in Supplemental Table 1. Incubation times were 2 h at room temperature for primary and 1 h at RT for secondary antibodies. Hoechst 33258 (2 μg/ml, Sigma) was added during secondary antibody incubation to counterstain cell nuclei. Negative controls were incubated with the secondary antibody only. Images were acquired with an inverted microscope Olympus IX71 (Carl Zeiss, Jena, Germany) equipped with a Hamamatsu ORCA-ER digital camera (Hamamatsu Corp., Bridgewater, NJ).

Histology and immunohistochemistry of the umbilical cord

Tissue was fixed in 4% PFA prior to embedding in OCT and cryosectioning for histological and immunohistochemical evaluation. Sections of umbilical cord were stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, post incubation in primary antibody overnight at 4 °C and washing 3 times with PBS, the sections were incubated in biotinylated secondary antibody (primary and secondary antibodies are listed in Supplemental Table 1) for 1 h. The target antigen signal was detected with Vectorstain Avidin–Biotin Complex kit (Vector Laboratories, Burlingame,CA). Hematoxylin was used to counterstain cell nuclei. Images were acquired with an Axiovert 135 (Zeiss) with a ProgRes C14 digital camera using OpenLab software (PerkinElmer Life).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (RT-qPCR)

RNA was extracted from cells and tissue using Tri-Reagent (Life Technologies) according to the manufacturer's protocol. RNA was retro-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI).

Real time quantitative polymerase chain reaction (RT-qPCR) was preformed with ABI Prism 7500 sequence detection system (Applied Biosystems) using the Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) following the

manufacturer's instructions. List of the used primers is summarized in Supplemental Table 3. Gene expression data were normalized using GAPDH housekeeping gene as a reference using the 2-ΔΔCt method.

Statistical analysis

Data are presented as mean ± SEM. The statistical analysis was performed using GraphPad Prism version 5.00 for Windows. Statistical significance was evaluated by Student's *t*-test or ANOVA followed by a Bonferroni post-hoc test. A p value equal to or less than 0.05 was considered as statistically significant.

Results

Comparison of surface and neural markers in UC-MSCs, p-ADSCs and NSCs

All three human somatic stem cell types, UC-MSCs, p-ADSCs and NSCs, possessed adherent properties and fibroblastic morphology. We assessed the expression of a typical panel of mesenchymal surface markers and neural markers in order to establish whether they could be readily distinguished on the basis of their surface markers and which human mesenchymal stem cell population, p-ADSCs or UC-MSC, shared more features with NSC. At least three cell lines from different individuals were used unless otherwise stated.

Mesenchymal surface markers were analyzed by flow cytometry in NSC, p-ADSC and UC-MSC (Table 1). We compared surface markers in UC-MSCs cultured under standard conditions (FBS-containing medium; UC-MSC–FBS) and human cord plasma-containing medium (UC-MSC–CP) to assess the potential usage of the latter as a substitute for FBS and ensure that the cellular profiles were not unfavorably changed. All cells expressed similar high levels of the mesenchymal markers CD29 (integrin β1), CD44 (hyaluronic acid receptor), CD73 (ecto-5′-nucleotidase), CD90 (Thy1) and CD166 (activated leukocyte cell adhesion molecule). NSCs tended to have a lower expression of CD105 (endoglin) compared to the other stem cell types, although only two separate NSCs isolates were analyzed for this marker. All cells expressed similar low levels of the negative markers CD14 (monocyte differentiation antigen), CD31 (platelet endothelial cell adhesion molecule-1), CD45 (lymphocyte common antigen) and CD117 (c-kit). High expression of CD10 (neprilysin) was noted in UC-MSCs cultured in CP ($p < 0.001$ vs. NSC, p-ADSC and UC-MSC–FBS); CD10 is a metallopeptidase typically expressed by hematopoietic progenitors with the capacity to differentiate into T, B or Natural Killer cells (Galy et al., 1995). NSCs were the only stem cell type with high expression of CD34 (hematopoietic progenitor cell antigen; $p < 0.0001$ vs. p-ADSC, UC-MSC–FBS and UC-MSC–CP); this was the main notable difference between NSCs and the mesenchymal stem cells in mesenchymal marker expression.

The expression of proteins normally associated with the neural lineage was assessed in UC-MSC–FBS, UC-MSC–CP and NSC by flow cytometry (Fig. 1, Table 2) as neurogenic markers in p-ADSCs had been previously characterized (Guasti et al., 2012). NSCs were taken as the positive control and used as a reference for the statistical analysis. The markers of neural stem/progenitor cells, Sox2, nestin, and vimentin, that are expressed early in development of the central nervous system, and prominin (CD133), a marker of neurogenic radial glia, were highly expressed in NSC, as expected. CD133, while found in 75% NSCs, was hardly detectable in UC-MSCs, independently from the culture conditions used ($p < 0.0001$). In contrast, vimentin was more highly expressed in UC-MSC–CP than UC-MSC–FBS ($p < 0.05$), with expression levels similar to those detected in NSCs (99%). No statistically significant difference was observed in the percentage of nestin-expressing cells among the different cultures. Expression of SOX2 was much lower in UC-MSCs than in NSCs ($p < 0.0001$); surprisingly, however, the UC-MSCs cultured in CP expressed higher levels of SOX2 protein compared to those

Table 1 Comparative characterization of NSC, UC-MSC and p-ADSC using flow cytometry.

		UC-MSC–FBS	UC-MSC–CP	p-ADSC	NSC
Mesenchymal markers	CD13	97.48 ± 1.4	99.93 ± 0.06	97.43 ± 2.16	82.07 ± 9.53 *
	CD29	99.28 ± 0.51	99.97 ± 0.03	98.70 ± 1.15	99.90 ± 0.10
	CD44	98.98 ± 0.80	99.95 ± 0.05	99.87 ± 0.03	99.83 ± 0.06
	CD73	98.28 ± 1.01	99.93 ± 0.06	99.17 ± 0.68	99.47 ± 0.33
	CD90	98.95 ± 0.85	99.95 ± 0.05	99.07 ± 0.23	67.13 ± 24.65
	CD105	99.00	98.28 ± 0.66	92.43 ± 6.18	57.20
	CD166	97.00 ± 1.55	99.67 ± 0.31	92.30 ± 6.45	99.50 ± 0.15
Non-mesenchymal markers	CD10	6.00 ± 5.80	82.32 ± 14.85 **	6.17 ± 2.4	0.18 ± 0.10
	CD14	0.57 ± 0.26	0.34 ± 0.21	0.52 ± 0.21	0.19 ± 0.15
	CD31	0.50 ± 0.14	2.17 ± 1.90	0.49 ± 0.30	4.94 ± 4.77
	CD34	1.43 ± 0.75	0.86 ± 0.22	2.42 ± 2.33	94.10 ± 2.04 ***
	CD45	0.34 ± 0.12	0.53 ± 0.12	0.58 ± 0.24	0.51 ± 0.17
	CD117	3.69 ± 1.41	1.26 ± 0.87	1.07 ± 0.18	10.0 ± 7.75

Data are percentages represented as mean ± SEM, $n \geq 3$; SEM is not shown when $n < 3$. Abbreviations: p-ADSC, pediatric adipose-derived stem cells; NSC, neural stem cells; UC-MSC, umbilical cord mesenchymal stem cells; CP, cord plasma; FBS, fetal calf serum.

* NSC vs. UC-MSC–CP $p < 0.05$.

** UC-MSC–CP vs. NSC, p-ADSC and UCSC–FBS $p < 0.001$.

*** NSC vs. ADSC, UC-MCS–FBS and UC-MSC–CP $p < 0.0001$.

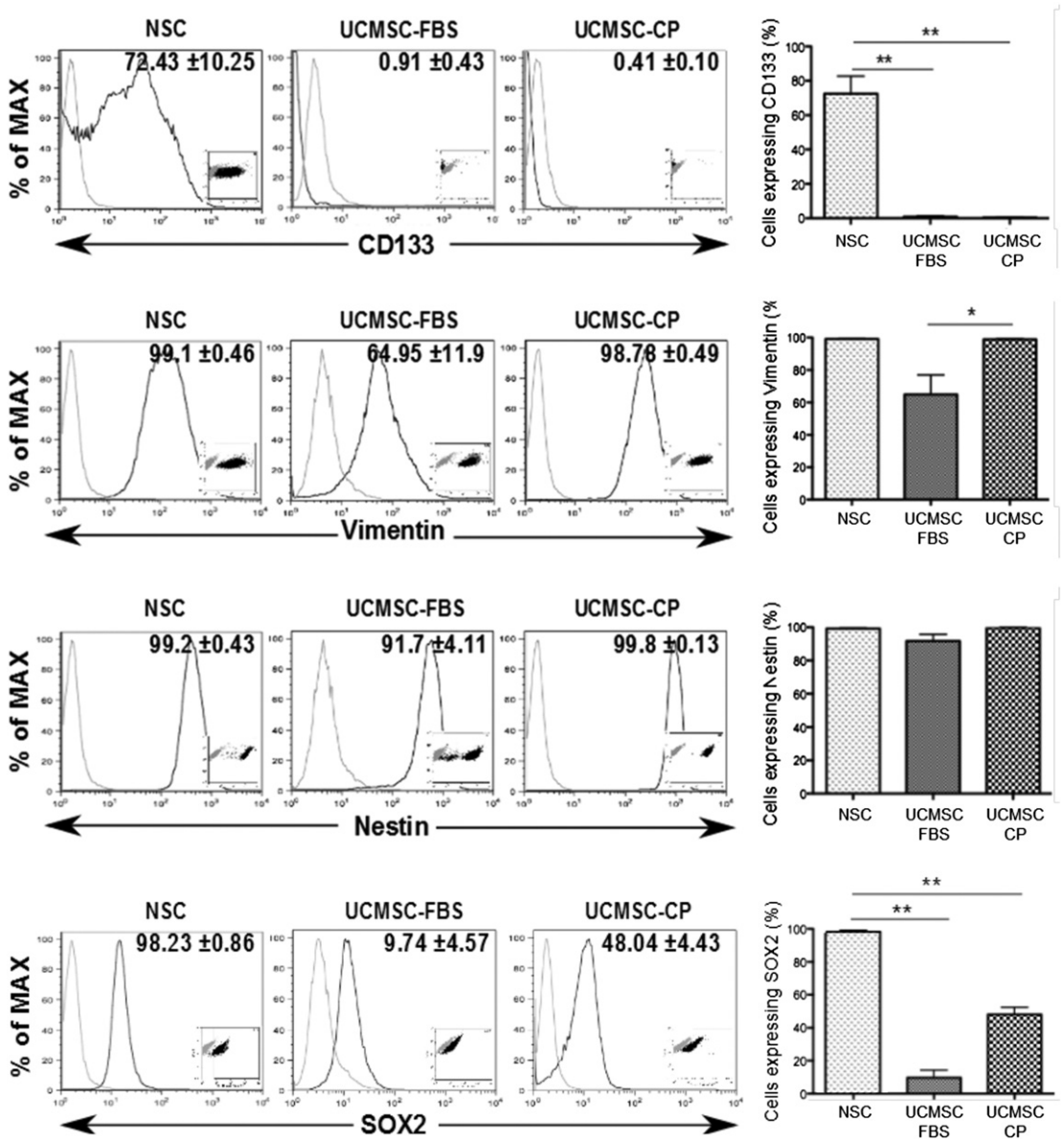


Figure 1 Comparative characterization of neural stem cell markers expressed by human UC-MSC (UCMSC) and p-ADSC (ADSC). Neural stem cell marker expression as detected by flow cytometry: CD133, vimentin, nestin and SOX2 protein expression of NSC, UC-MSC–FBS, and UC-MSC–CPs. Histograms/plots: gray line(–)/plots = negative control; black line(–)/plots = sample. Dot plot gate: positive/negative threshold. Shown one representative out of ≥3 independent experiments. Number = % of cells represented as mean ± SEM. Graphs show statistic comparison of protein expression between populations. *$p < 0.05$; **$p < 0.001$. Abbreviations: UC-MSC, umbilical cord mesenchymal stem cells; FBS, fetal bovine serum; CP, cord plasma; NSC, neural stem cells.

cultured in FBS ($p < 0.001$), though the percentage of SOX2-positive UC-MSC–CB (48%) was not as high as in NSCs (98%). As SOX2 is expressed also in pluripotent human embryonic stem (ES) cells, we wished to establish whether expression of SOX2 in UC-MSC–CB might be associated with a reversal to pluripotency rather than reflecting neural potential. Analysis of the expression of the ES cell markers, SSEA-1, SSEA-4, and OCT3/4, by flow cytometry showed that SSEA-1 was not expressed in UC-MSC–CPs, and that less than 6% of cells expressed SSEA-4, and OCT3/4 (Fig. 2). Analysis of OCT4 by RT-PCR confirmed lack of expression of this pluripotency transcription factor. This suggests that up-regulation of SOX2 is not associate with an ES-like phenotype.

To further confirm this, we assessed expression of additional neural markers that had been detected in p-ADSCs (Guasti et al., 2012). As shown by flow cytometry, surprisingly, the neuronal marker, ß3-tubulin, was similarly expressed in UC-MSC-CPs and NSCs. In contrast, low to nil expression of GFAP, a marker of neurogenic radial glia detected in 66% of the NSCs, was observed in the UC-MSCs independently of the culture conditions used. The oligodendrocyte progenitor marker, O4, was not detected in any of the cell lines, but PDGFRα, that has been associated with motility of mesenchymal stem cells, as well as being a very early oligodendrocyte precursor marker, was expressed in high percentage of cells (77%) only in the UC-MSC–CP cultures. The late neural markers

Table 2 Comparative characterization of neural lineage-associated markers expressed by NSC and UC-MSC using flow cytometry.

	NSC	UC-MSC−FBS	UC-MSC−CP
GFAP	66.40 ± 3.85 †	2.03 ± 0.93	1.63 ± 0.64
PDGFRα	0.70 ± 0.41 ††	2.88	77.32 ± 11.30
O4	0.38 ± 0.24	0.96 ± 0.58	0.18 ± 0.04
P75NGFR	12.46 ± 4.46 ‡	1.34 ± 0.92	0.76 ± 0.15
β3-Tubulin	99.50	92.87 ± 3.93	95.30 ± 0.42

Data are percentages represented as mean ± SEM, n ≥ 3, if no SEM is indicated only two isolates were analyzed (n = 2). Abbreviations: UC-MSC, umbilical cord mesenchymal stem cells; FBS, fetal bovine serum; CP, cord plasma; NSC, neural stem cells, *N/P*, non-performed.

† NSC vs. UC-MSC−FBS and UC-MSC−CP $p < 0.0001$.

‡ NSC vs. UC-MSC−FBS and UC-MSC−CP $p < 0.001$.

†† NSC vs. UC-MSC−CP $p < 0.05$.

P75 NGFR (CD271), recently identified also in human neural progenitors in the adult subventricular zone, was hardly detectable in UC-MSC−CPs, and present only in a small subset of NSCs (12%).

To assess cellular localization of neural markers detected in UC-MCS by flow cytometry, and establish whether expression of such markers in UC-MSCs might reflect the existence of a sub-population of cell in the Wharton's jelly (Fig. 3A) from which they were derived, we carried immnocytochemistry experiments, UC-MSCs, like the Wharton's jelly in cord sections, expressed vimentin, and approximately 50% of the cells in culture were smooth muscle alpha actin (αSMA)-positive (Figs. 3B−E). In contrast, neural markers expressed by UC-MSCs, including nestin, GFAP, β3-tubulin (Figs. 3F−H) and NF-200 (not shown) were not detected in the Wharton's jelly (not shown). The expression of the cytoskeletal neural markers, GFAP, β3-tubulin and NF-200, was non-filamentous in the UC-MSCs. In addition, low levels of SOX2 expression were observed in UC-MSC cytoplasm, but not in the umbilical cord sections (not shown). No reactivity was noted in negative controls (Fig. 3I).

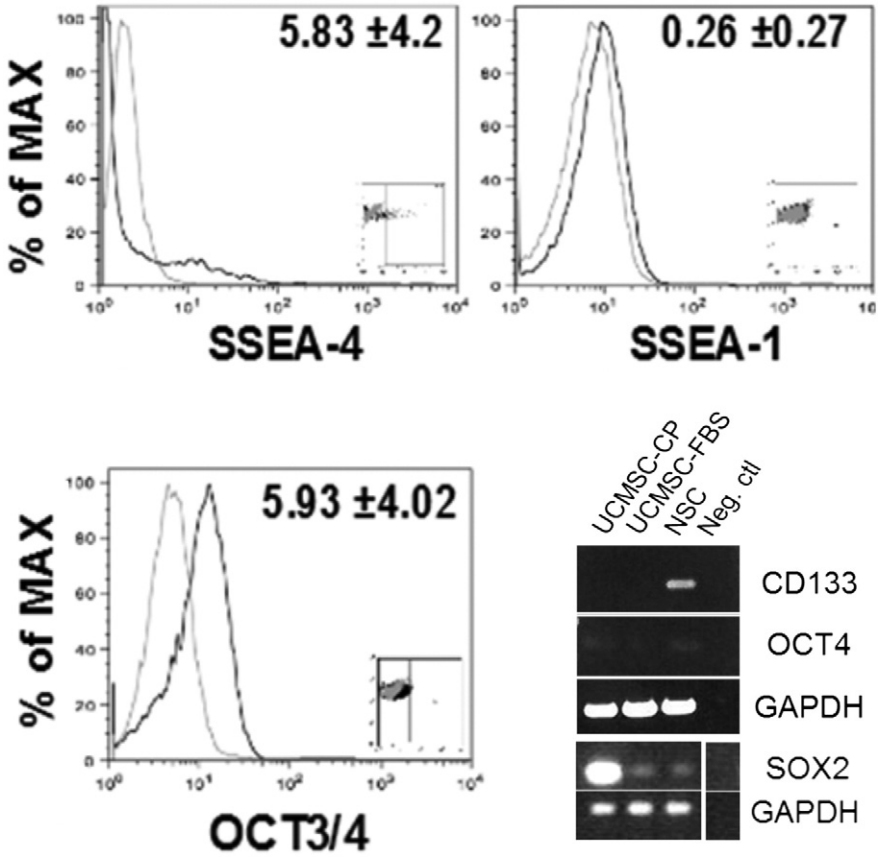


Figure 2 Pluripotency marker expression as detected by flow cytometry and RT-PCR: SSEA-4, SSEA1, and OCT3/4 protein expression in UC-MSC-CPs. Histograms/plots: gray line(−)/plots = negative control; black line(−)/plots = sample. Dot plot gate: positive/negative threshold. Shown one representative out of ≥3 independent experiments. Number = % of cells represented as mean ± SEM. Graphs show statistic comparison of protein expression between populations. *$p < 0.05$; **$p < 0.001$. CD133, OCT4 and SOX2 gene expression in UC-MSC−FBS, UC-MSC−CP and NSC in comparison with the gene expression of the housekeeping gene, GAPDH. Abbreviations: UC-MSC, umbilical cord mesenchymal stem cells; FBS, fetal bovine serum; CP, cord plasma; NSC, neural stem cells.

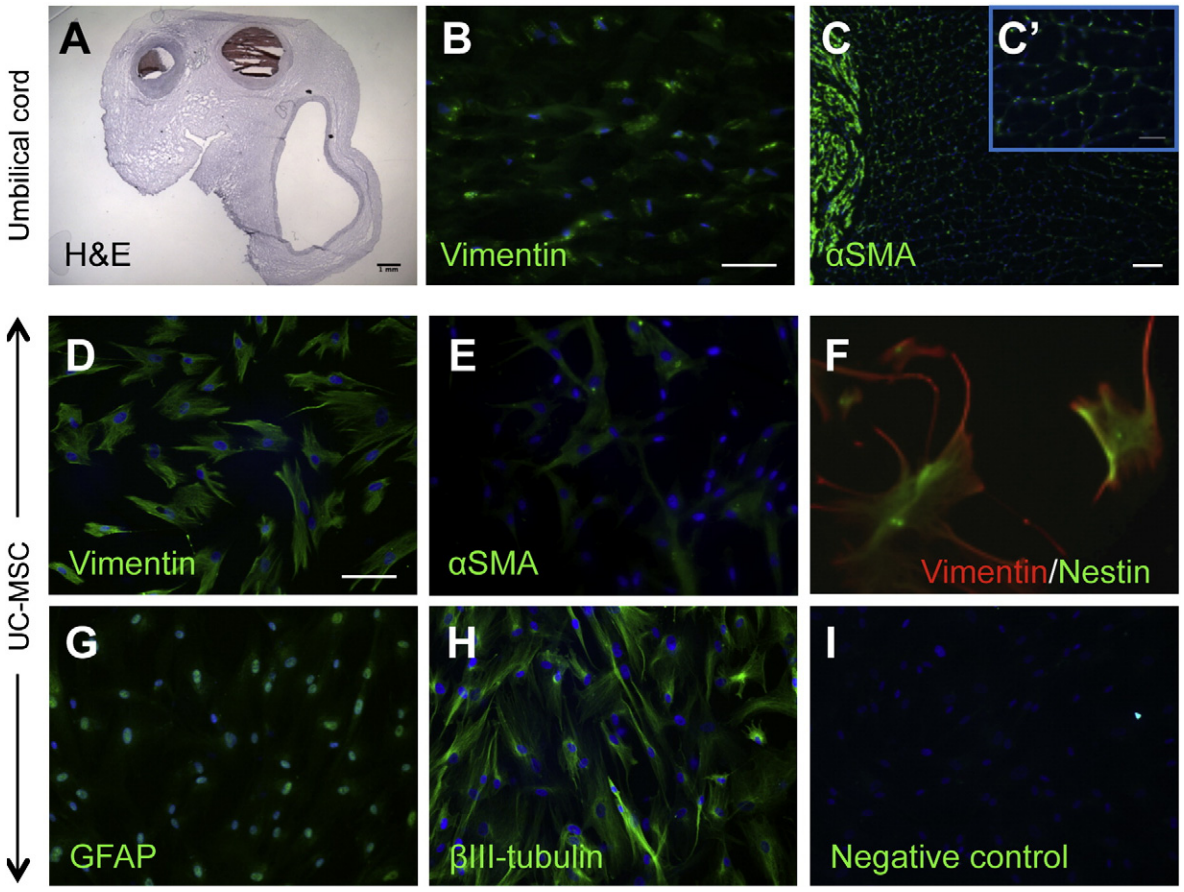


Figure 3 Protein expression in human umbilical cord sections and in human umbilical cord-derived mesenchymal cells (UC-MSC) assessed by immunochemistry (green). A–C) Human umbilical cord cross sections stained with hematoxylin and eosin (A), and by immunohistochemistry for vimentin (B) and αSMA (C) shown at high magnification in the inset (C′). D–I) Expression of mesenchymal and neural proteins in UC-MSC detected by immunocytochemistry: vimentin (D), αSMA (E), nestin (F), GFAP (G) and βIII-tubulin (H); negative control where the primary antibody was omitted (I). Nuclei are visualized with Hoechst 33258 (blue). Scale bars = 100 μm. Abbreviations: H&E, hematoxylin and eosin; αSMA, alpha smooth muscle actin; GFAP, glial fibrillary acidic protein.

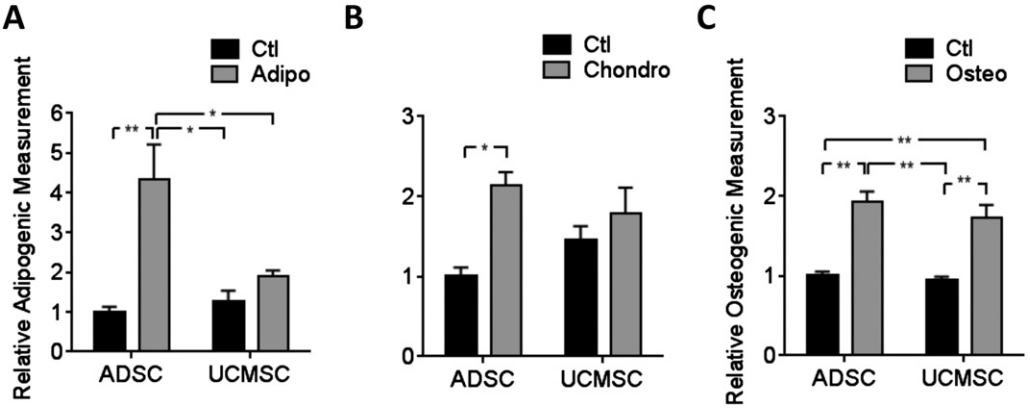


Figure 4 Comparison of the tri-lineage differentiation potential of UC-MSC and p-ADSC. A) Quantification of adipogenesis (oil Red O quantification). C) Quantification of chondrogenesis (alcian blue quantification). D) Quantification of osteogenesis (Alizarin Red quantification). E) Data are expressed as fold changes, taking untreated p-ADSC controls as 1; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: p-ADSC, pediatric adipose-derived stem cells; UC-MSC, umbilical cord mesenchymal stem cells.

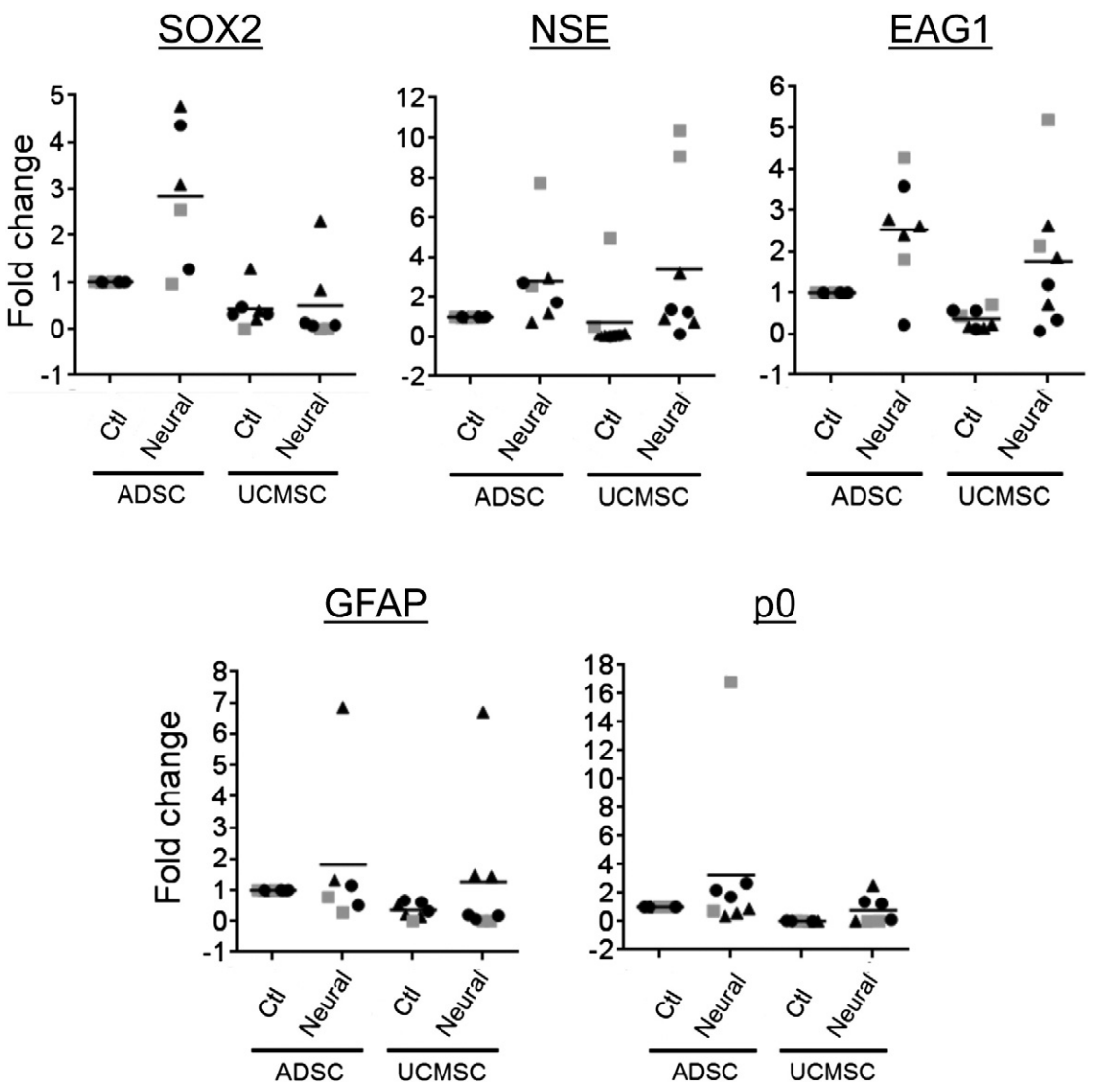
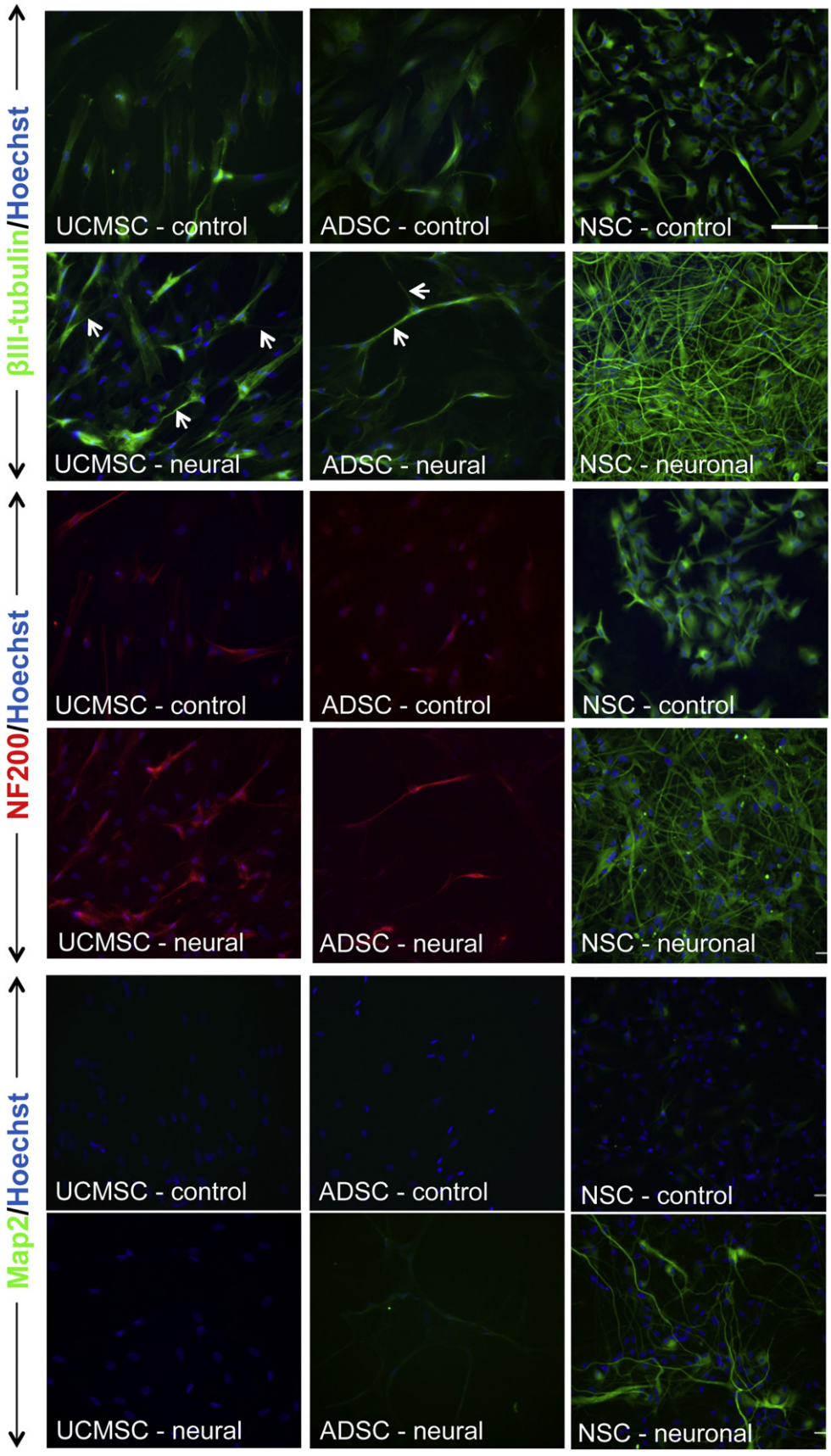


Figure 5 Neurogenic potential of UC-MSC and p-ADSC. RT-qPCR analysis of neural differentiation markers in cells maintained either in control or neurogenic medium, including the early neural stem cell marker, SOX2, the neuronal markers, NSE and EAG1, and the glial markers, GFAP and p0. Data are expressed as fold changes, taking untreated p-ADSC controls as 1. Abbreviations: UC-MSC, umbilical cord mesenchymal stem cells; p-ADSC, pediatric adipose-derived stem cells; NSE, neuron-specific enolase, EAG1, ether-à-go-go; p0, myelin protein zero; GFAP, glial fibrillary acidic protein.

Differentiation potential of UC-MSCs and p-ADSCs

The plasticity of UC-MSCs and p-ADSCs was compared by assessing their potential to differentiate towards mesenchymal lineages (chondrogenic, adipogenic and osteogenic) when cultured in the same medium. Three independent cell lines were used for all experiments. Both UC-MSCs and p-ADSCs could differentiate towards the three mesenchymal lineages as indicated by semi-quantitative analysis of oil red, alcian blue and alizarin red staining to detect adipogenic, chondrogenic and osteogenic differentiation, respectively (Fig. 4). UC-MSCs less readily underwent adipogenic and chondrogenic differentiation when compared to the p-ADSCs ($p < 0.01$ and $p < 0.05$ respectively). The extent of osteogenic differentiation was comparable in both UC-MSCs and p-ADSCs; a statistically significant difference was noted when compared to the controls ($p < 0.01$).

Figure 6 NSC, UC-MSC and p-ADSC neuronal differentiation potential. Protein expression of neuronal markers in undifferentiated (control) and differentiated NSC, UC-MSC and p-ADSC as detected by immunofluorescence. Positive expression in green and nuclei are visualized with Hoechst 33258 (blue). (B–I) Scale bars = 100 μm. Abbreviations: NSC, neural stem cells, UC-MSC, umbilical cord mesenchymal stem cells; p-ADSC, pediatric adipose-derived stem cells; TuJ1, βIII-tubulin, NF200, neurofilament-200; MAP2, microtubule-associated protein 2.


βIII-tubulin/Hoechst
NF200/Hoechst
Map2/Hoechst
UCMSC - control
ADSC - control
NSC - control
UCMSC - neural
ADSC - neural
NSC - neuronal

Following exposure of p-ADSCs and UC-MSCs to neurogenic induction media (Fig. 5), a general trend in up-regulation of neural markers was noted in both p-ADSCs and UC-MSCs, although variability between individual lines of each cell type was noted (three were analyzed per cell type). SOX2 transcript was up-regulated to a larger extent in UC-MSCs than p-ADSCs. Neuronal marker transcripts, the neuron-specific enolase (NSE) and the voltage-gated potassium channel *EAG1* (ether a-go-go-1), were increased in both p-ADSCs and UC-MSCs. There was little increase in mRNAs encoding for glial markers, such as glial fibrillary acidic protein (GFAP), and the Schwann cell marker, P0, in both cell types. Up-regulation of neuronal markers at the transcriptional level in UC-MSCs and p-ADSCs, supports their potential to differentiate towards both lineages.

We then further investigated the neurogenic potential of these cells and evidence of differentiation at the morphological and protein level. Protein expression of the neuronal markers, NF-200 and βIII-tubulin, was assessed by immunocytochemistry in UC-MSCs and p-ADSCs differentiated in parallel, and compared to NSCs differentiated using the same forskolin-containing induction media (Fig. 6). The morphology of both mesenchymal stem cells was dramatically changed after two weeks in neurogenic induction media, with differentiated p-ADSCs forming spindly networks unlike that of the control p-ADSCs and closer to that of the differentiated NSCs. The non-induced UC-MSC expressed neuronal markers on a basal level, similar to that shown previously for p-ADSCs (Guasti et al., 2012). However some increase in NF-200 and βIII-tubulin expression was noted in UC-MSCs and p-ADSCs cultured in the neuronal differentiation medium. Low levels of MAP2 expression were detected in differentiated p-ADSCs, but not in controls nor in differentiated UC-MSCs. The differentiated NSC positive controls highly expressed all three neuronal markers.

Discussion

To the best of our knowledge, this is the first study to compare in parallel a set of surface markers in the human somatic stem cells, p-ADSCs and UC-MSCs, with NSCs. The prototypical feature of MSCs is said to be their expression of a defined set of surface markers including CD73, CD90 and CD105 (Kim et al., 2013). The MSCs analyzed here, despite having been isolated from different tissues and displaying some variations in their differentiation potential, did not exhibit any discernible difference in their surface marker expression, consistent with other reports (Wagner et al., 2005; Hu et al., 2013). However, as shown by this study, NSCs also display these surface markers, as well as other so-called mesenchymal markers. On the other hand, the mesenchymal stem cells studied here were shown to express "neural" markers. This highlights the challenge of distinguishing between cell types and may explain discrepancy in results from different laboratories. Crucially, it raises important issues concerning the minimal number and combination of markers required for accurate phenotyping of somatic progenitor/stem cells.

Neural markers are expressed in human mesenchymal stem cells and non-neural markers in neural stem cells

Constitutive expression of neural markers has been previously reported in p-ADSCs by us and in human bone marrow-derived MSC by other groups (Tondreau et al., 2004). A subset of nestin-positive cells has also been observed in UC-MSCs (Weiss et al., 2006), but their percentage (23%) was much lower than in our cultures (between 92 and 100% in both growth media used). The presence of nestin-positive cells in UC-MSC cultures could be taken to reflect selection in vitro of perivascular cells akin to those described in the bone marrow stem cell niche (Mendez-Ferrer et al., 2010; Pacini and Petrini, 2014). This interpretation, however, is not supported by analysis of umbilical cord sections, where no evidence of nestin expression was noted. Furthermore, there was no immunoreactivity for βIII-tubulin in the umbilical cord, though this protein was found to be expressed by the UC-MSCs using both FACS analysis and immunofluorescence; βIII-tubulin cellular organization in these cells, however, was not as filamentous as in neurones. Notwithstanding βIII-tubulin being defined as a neuronal marker, its expression in human mesenchymal cultures has also been reported in human p-ADSCs by us and in bone marrow-derived MSCs by others (Guasti et al., 2012; Tondreau et al., 2004). Given that in human tissue sections, βIII-tubulin staining appears to be neural-specific and is not found in mesenchymal tissues, it has to be concluded that βIII-tubulin neural-specificity in humans is context dependent, and its expression is induced in human cells with progenitor/stem cell potential when grown in vitro. To our knowledge, there are no reports of this protein being expressed in vitro in a range of somatic stem cells in other species. The significance of this difference is not currently clear, but this is bound to change as differences as well as similarity between mouse and human cells gradually become better understood (Carninci, 2014).

Together, it seems unlikely that the in vitro conditions select for a small subset of isolated UC-MSCs that possess a neural phenotype; our results rather suggest that differences in culture conditions induce changes in cell phenotype that may reflect their intrinsic plasticity and potential to be induced along different lineages. This is supported also by differences in expression of SOX2, PDGFR and CD10 in UC-MSCs grown in different media. CD10 expression is indeed known to vary between MSCs isolated from different sources and appears to be dependent on serum or growth factors used (Mariotti et al., 2008).

The only surface markers we found to be differentially expressed between mesenchymal and NSCs were CD133 and CD34, that were detected only in the latter. Expression of CD133 in NSCs was expected, having been observed in neural stem cells in both human and mouse; however it should be noted that other stem cell types, including endothelial, hepatic and hematopoietic stem/progenitor cells, express CD133 (Bhatia, 2001; Reubinoff et al., 2001; Schmelzer et al., 2006; Sousa et al., 2014; Urbich and Dimmeler, 2004). In contrast, we were surprised that the majority of NSCs expressed CD34, widely accepted to be a hematopoietic stem cell marker, but with as yet poorly defined function.

Cells positive for CD34 have been detected also in embryoid-like bodies (Reubinoff et al., 2001) and a member of the CD34 family is widely expressed in the developing mouse brain (Nielsen and McNagny, 2008; Vitureira et al., 2005; Nowakowski et al., 2010). Whether expression of CD34 reflects a yet unknown role of this membrane protein in NSCs, or a leaky and/or plastic phenotype of these cells that become apparent in culture remains to be established. Comparison of hNSC subpopulation growth and differentiation potential will help to address some of these issues.

Our study clearly shows that expansion media represent a significant variable when phenotyping cells. In our experiments we used the same ES-qualified serum to expand p-ADSCs and UC-MSCs from different individuals for direct comparison of their properties and differentiation potential. However, we used a single CP, a platelet-poor human cord plasma supplemented with growth factors to speed their otherwise slow expansion rate, as the aim was to assess whether UC-MSCs from different cords responded similarly to the human medium. While our results demonstrated that UC-MSCs could be effectively expanded in CP, it is not clear at this stage whether different CBs will differently affect gene expression and differentiation potential of UC-MSCs. Hence, while the use of human serum would in principle simplify taking any future stem cell therapy from bench to bedside (Spees et al., 2004), it may also increase variability in UC-MSC behavior. More studies are needed to clarify the benefits and limits of this xenogeneic-free serum and its role during expansion–differentiation protocols as compared to well characterized and standardized fetal bovine sera.

UC-MSCs have more limited differentiation potential than pediatric ADSCs

We have shown here that p-ADSC more readily differentiated towards the chondrogenic and adipogenic lineages than UC-MSCs, whereas a similar osteogenic potential was noted for both types of cells. This is consistent with the findings by Hu et al. who compared adult ADSCs and UC-MSCs, and supports the use of p-ADSCs for skeletal tissue engineering in young patients and in particular for cartilage reconstruction.

We performed side-by-side experiments with p-ADSC to compare the neurogenic potential of these mesenchymal cell types in parallel. A general trend was noted in our study, in which an increase in neuronal-specific transcripts was seen for both p-ADSCs and UC-MSCs to a similar extent when placed in a forskolin-containing media, demonstrating the capacity/potential of these cells to acquire phenotypic characteristics not only of mesenchymal but also of neuronally differentiated cells. This is consistent with various reports noting the potential of UC-MSC to differentiate towards neural lineages (Dalous et al., 2013; Divya et al., 2012); nonetheless, differences between cell lines and individual samples were apparent, which may account for the variability in results/conflicting results seen between different studies. Despite the similarities at a basal level in neural gene expression between the two mesenchymal cell types, p-ADSCs appear to respond more to the neuronal differentiation medium used here in regard to morphological changes, that suggested acquisition of neurone-like bipolar morphology, as well as to up-regulation of neuronal transcripts and proteins. Some expression of MAP2 was noted only in induced p-ADSCs. However, it should be stressed that refinement of the differentiation protocols or additional steps in the protocol will be needed to obtain bona fide mature functional neurones from either of these mesenchymal cell types. Definitive evidence of neural trans-differentiation requires assessment of neurological properties such as neuronal polarity, synapse formation, and electrophysiological characterization (Croft and Przyborski, 2006). Although several encouraging studies have produced electrophysiological data as well as appropriate gene transcript up-regulation from neuronally-differentiated ADSC (Ashjian et al., 2003; Anghileri et al., 2008; Jang et al., 2010), as yet no study has been able to demonstrate the trans-differentiation of these cells into a mature fully-functional neurone. This may prove difficult without recurring to gene manipulations; however the benefit of being able to use non-genetically modified autologous somatic cells, instead of allogeneic cells or iPS-derived cells, makes it important to strive towards this goal.

Conclusions

The differences in terms of markers and differentiation potential between somatic stem cell types – be it hematopoietic, mesenchymal or neural – appears to be narrowing with our ever expanding knowledge, emphasizing the need to develop better means of distinguishing between the different classes. The lack of surface marker combinations highly specific to each cell type and the effect of expansion media on the phenotype is of particular concern and needs to be resolved, in particular for development of cell-based therapies and appropriate interpretation of in vivo outcomes.

Direct comparison of UC-MSCs and p-ADSCs has shown that UC-MSCs have a more limited differentiation potential along multiple lineages, indicating that p-ADSCs will provide a more suitable cell source particularly for autologous cartilage reconstruction in children.

Finally, while this study has provided encouraging data on the neurogenic potential of the human somatic cells studied after only a few weeks of differentiation, it is important to note that much additional work is needed to establish whether a more mature neural phenotype from either of the mesenchymal stem cell population studied here can be induced without genetic manipulations.

Authors' contribution

SN designed and performed experiments, analyzed data and wrote the manuscript; CAG and BV designed and performed experiments, analyzed data and contributed to the writing; AM, SG and NWB provided reagents, tissues and critical reading of the manuscript; PF planned research, analyzed data, obtained funding, and wrote the manuscript.

Acknowledgments

This work was supported by grants from the Newlife Foundation (grant 12-13/20), the Great Ormond Street Hospital Children's Charity (grant V1234), the Antony Nolan Trust, and a studentship to CAG from Consejo Nacional de Ciencia y Tecnología (CONACyT, grant 210924) and Instituto Jaliscience de la Juventud (IJJ, grant FOLIO 150); Mexico. The human embryonic and fetal material was provided by the Human Developmental Biology Resource (http://hdbr.org) jointly funded by the Medical Research Council (grant G070089) and The Wellcome Trust (grant GR082557).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.04.003.

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